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An in Vitro Analysis of the Population Dynamics of Hemophilus Influenzae in Combined Infection With Influenza Virus.

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AN IN VITRO ANALYSIS OF THE POPULATION DYNAMICS OF HEMOPHILUS INFLUENZAE IN COMBINED INFECTION WITH INFLUENZA VIRUS

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ABSTRACT

This experimental study is an application of *in vitro* methods to an analysis of some of the factors that enhance the growth rate and virulence of *Hemophilus influenzae* in combined infection with influenza virus in embryonated eggs. The experiments were designed to determine whether influenza virus itself or certain substances derived from virus infected cells constituted the enhancing factors.

The basic media in which *Hemophilus* was cultured under appropriate comparable conditions were of four types. Levinthal's broth only (L) the generally accepted culture media of choice for *Hemophilus* constituted the control and basis for comparison. Levinthal's broth with equal parts of amniotic fluid from influenza virus infected embryonated eggs (V), Levinthal's broth with equal parts of amniotic fluid from infected eggs from which the virus had been removed by hemagglutination (SO) and Levinthal's broth with equal parts of amniotic fluid from normal uninfected embryonated eggs (N) were the other three media used.

Data for constructing growth curves of Hemophilus in these media were obtained by colony counts of appropriately diluted samples drawn at two hour intervals from the cultures during the first day of incubation at 37° C. The analysis of the population composition of Hemophilus was obtained by measuring the precipitinogen producing capacity of thirty random colonies grown out on Levinthal's agar from measured samples removed at three hour intervals during the early growth period.

Multiplication of Hemophilus took place most rapidly and in approximately equal rate in media (V) and (SO). This indicated in the first place that the removal of the virus from amniotic fluid from infected embryos, did not influence the growth rate of Hemophilus appreciably. Thus it could be concluded that the virus particles per se did not substitute a growth enhancing factor. The addition of normal amniotic fluid increased the growth rate as compared to the normal broth control but to a considerably less extent than the addition of amniotic fluid from virus infected embryos.

In media (V) and (SO) the proportion of virulent (precipitinogen producing) clones of Hemophilus increased during the first twelve hours of growth as compared with growth in media (B) in which the proportion of virulent clones decreased. Thus it appears that the same factors that are responsible for the more rapid growth rate also apparently

maintain or enhance the virulence of the bacterial population in combined infection. The virus particles *per se* do not seem to possess these enhancing influences.

The methods developed in these studies are applicable to a further exploration into a more exact characterization of the substances produced by virus infected cells that serve to enhance bacterial virulence. Preliminary tests indicate that these are protein in nature and that their separation and identification may be accomplished.

INTRODUCTION

The problem of combined viral and bacterial infection in the causation of disease originated during the influenza pandemic of 1918. It became apparent at that time that the serious and often fatal pneumonias due to different pyogenic bacteria were usually secondary infections superimposed on a primary process caused by what then was referred to as a filterable virus. The experimental approach to an analysis of this phenomenon began with Shope's demonstration of the synergistic role of swine influenza virus and Hemophilus influenzae var. suis in swine influenza. During the past thirty years a considerable number of experimental investigations of this problem have been conducted with strains of human influenza type A virus and various bacterial species in different host groups under a variety of more or less controlled circumstances. In general, this has led to the conclusion that the injury and necrosis of susceptible cells produced by the initial virus infection provided conditions and substances in which pathogenic bacteria thrive and become the cause of the secondary pneumonia.

This rather crude explanation might account for what happens in epidemic influenza where considerable damage to

respiratory tract epithelium takes place. It is not apparent what is responsible for the enhancement of bacterial infections complicating a milder viral respiratory disease in which no severe injury or damage can be demonstrated. This became clear in a series of studies carried out over the past ten years in this laboratory. Influenza type C which does not produce detectable injury or disease in embryonated eggs served as the initial viral component in combined infection with Hemophilus influenzae type b, as the bacterial component. In this experimental model, a careful analysis of the infectious process indicated that the observed enhancement of virulence of the bacterial element in the final infection posed problems relating to population dynamics. It was evident that in the virus infected embryonated egg, the bacterial population not only multiplied more rapidly, but that the proportion of virulent elements in this population was maintained or even partly enhanced. Under these circumstances, greater numbers of virulent and more invasive bacteria were available during the very early stages of the infectious process than in the absence of viral infection in its mildest form.

These in vivo experiments did not lend themselves to a more precise analysis of the factors responsible for the enhancement of bacterial virulence in the combined infections. In vitro methods were devised in order to determine

whether the viral component *per se* or whether substances derived from virus infected cells constituted the factors that promoted enhancement of virulence. The results and observations made in these investigations and the conclusions drawn from them make up the subject matter of this dissertation.

REVIEW OF THE LITERATURE

In 1931, Shope (28) observed that Berkefeld filtrates of infectious material from cases of experimental swine influenza introduced intranasally into susceptible swine produced a mild febrile disease. When these filtrates were combined with cultures of H. influenzae suis and administered intranasally, a disease simulating influenza in its clinical and pathological features was initiated. This manifestation was not seen when either one of the two agents was introduced alone. He postulated that in the natural disease the bacterial component activated the latent viral infection. In 1930, Dochez, Shibley and Mills (10) transmitted to chimpanzees by means of filtrates of human throat washings what was considered to be the agent of the common cold. This was found to be followed by a sudden increase in the number of pneumococci in the throat of the experimental animal. In 1932, Dochez, Mills, and Kneeland (11) used the same procedure and they isolated H. influenzae from both the nasopharynx and the nasal cavities of chimpanzees with experimentally induced common cold. These authors observed that the smooth, more virulent form

of H. influenzae prevailed in chimpanzees with the experimental common cold, while the rough, less virulent form of the bacterium was the major isolate from animals which were apparently free from the virus. In 1936, Brightman (3, 4) observed that when nasal and/or throat washings from children suffering from spontaneous infection with influenza virus were inoculated intranasally into ferrets, a disease indistinguishable from influenza ensued. The severity of the disease was generally enhanced by serial transmission of the virus in ferrets. Although bacterial culture revealed the presence of H. influenzae in washings from the children, these were not recovered from the inoculated ferrets. During the early stages of the investigation he was able to isolate staphylococci, gram negative cocci, and diphtheroid bacilli from nasal washings of ferrets with influenza virus infection. However, after five serial passages of the virus in these animals, he found that Streptococcus hemolyticus (group C) was often present in the nasal washings. Furthermore, this micro-organism could be recovered by culture of the lungs, heart blood, and viscera of animals that died from the experimentally induced infections. He concluded that the hemolytic streptococci had been quiescent in the upper respiratory tracts of these animals and then multiplied freely when resistance was lowered by the viral infection.

In 1944, Schwab, Blubaugh, and Woolpert (26) observed a higher mortality and shorter survival time of mice infected with a mixture of Streptococcus hemolyticus (group C) and influenza FM virus than in those infected with either of these agents alone. In the same year Glover (16) observed that ferrets could only be infected with Streptococcus hemolyticus (group C) by intranasal instillation of the micro-organism when the animals were inoculated simultaneously or few days previously with influenza virus.

In 1945, Bang (1) reported what he called the "synergistic effect" of H. influenzae suis when combined with swine influenza virus in the chorio-allantoic membrane of 9-10 day chick embryos. This was maintained through at least 11 chick embryo passages of the virus. He also found that the combination of H. influenzae suis with swine influenza virus caused considerable destruction of embryo lungs; an effect that was not observed with either agent alone. Within the same year and in another investigation (2), he found that the addition of H. influenzae to the chorio-allantoic membrane of chick embryos infected with swine influenza virus caused the virus to spread from the membrane to the embryo proper. This spreading could be brought about also with a purified preparation of hyaluronidase. The latter was not accompanied by a comparable increase in mortality. In 1945, Francis and De Terregrosa (13) found that infection with

virulent forms of H. influenzae could not be established in the respiratory tract of apparently normal mice. Nevertheless, when the bacteria were introduced 1-5 days after establishing influenza type A virus infection, the mice died. H. influenzae had apparently multiplied so that it could be recovered from the lungs. To a lesser extent, the same effect was noted with Streptococcus hemolyticus. It appeared that in certain cases the virulence of the bacteria was enhanced by previously established viral infection. In 1946, Hartford, Smith, and Wood (17) observed the development of pneumonia in mice inoculated intranasally with sublethal doses of PR8 influenza virus followed by droplets of pneumococcal cultures. Normal mice did not develop recognizable disease by inhalation of the bacteria. In 1947, Wilson, Saslaw, Doan, Woolpert, and Schwab (29) found that monkeys with experimentally induced combined Streptococcus hemolyticus (group C) and PR8 influenza virus infection exhibited a more severe disease as compared to the infection with either of these agents alone. In 1947, Carlisle and Hudson [8, 9] produced mild infection in mice by intranasal instillation of 0.05 ml of influenza type A virus. At intervals of 0, 2, 4, 8, 12, 16, 24, and 32 days after viral inoculation, the susceptibility of these animals was tested by infectivity titration using group C Streptococcus hemolyticus in experimental and control groups. The virus infected mice

were found to be 100 times more susceptible than the controls to fatal infection at the 2nd, 4th, 8th, and 12th day. No marked differences were noted in mice receiving streptococci more than 12 days after the initial virus inoculation. Streptococci could be cultured from all fatally infected mice. Heat inactivated influenza virus had no effect on the susceptibility of mice to streptococcal infection. Heat killed streptococci did not change the course of the mild influenza virus infection. Harford, Leidler and Hara (18) (1948) concluded from their experiments that the lungs of normal mice are capable of markedly reducing the number of type I Pneumococcus within three hours after nasal instillation. However, in lungs with fully developed viral pneumonia, bacterial growth was markedly enhanced. In 1950, Harford and Hara (19) observed that mice with experimental pulmonary edema produced by viral infection, drugs, etc., developed increased susceptibility to inhaled pneumococci. Under these circumstances, the micro-organisms multiplied well in the lungs with a resulting bacterial pneumonia. These authors emphasized that the chief effect of the pulmonary edema was to furnish a more suitable fluid medium for the growth of pneumococci.

Conditions in human beings that are known to be complicated by pulmonary edema such as occur in respiratory viral infections are known also to be associated with an

apparent increased susceptibility to secondary bacterial infections. In 1953, Wood, Buddingh, and Abberger (30) reported an investigation of 51 cases of acute bronchiolitis in infants. They concluded that H. influenzae types a and b must be considered in the etiology and pathogenesis of this disease. They did not determine but suggested that other bacteria, viruses, or a combination of bacterial and viral agents were involved.

In 1956, Buddingh (5) published an extensive experimental study of combined viral and bacterial infection. Upon the introduction of influenza virus type C into the amniotic sac of 14 day chick embryos, no noticeable disease process developed. When 15 day embryos were infected with H. influenzae type b via the amniotic route, death, bacteremia or characteristic inflammatory lesions in the form of purulent sinusitis, pharyngitis, tracheo-bronchitis, and meningo-encephalitis could be detected in a certain proportion by microscopic study of appropriate sections. These lesions occurred singly or in various combinations. The incidence, pathogenesis, and severity of the manifestations were shown to be dependent on the proportion of the encapsulated virulent component of the inoculum. When this bacterial infection was superimposed 24 hours after the establishment of the virus in the embryos, the incidence and severity of the disease were significantly higher in

the combined infections than in those infected with the bacilli alone. It was suggested that infection with the viral component in the combined infection promoted the selective survival and more rapid multiplication of the more virulent elements in the bacterial population. In 1957, Gerone, Ward, and Chappell (14) encountered a higher mortality in albino mice infected with PR8 influenza virus and pneumococci than those with either of the two agents alone. They indicated that the inhibition of antibody formation to the virus and the enhancement of bacterial growth resulted in pneumococcemia.

In 1959, Martin, Kunin, Gottlieb, Barnes, Liu, and Finland (23) reported 32 fatal cases of influenza occurred in the Boston Area during the pandemic of 1957-1958. Fifteen cases were of the pure viral type, 11 of the post-influenza staphylococcal pneumonia, and 6 were influenza complicated with other types of bacterial invaders. In their attempts to culture bacterial agents from lung tissues, no pathogen was recovered from the cases of pure viral infections, while all the staphylococcal pneumonia victims revealed a large number of Staphylococcus aureus. Among the third group, only three cases, which showed Pneumococcus pneumoniae in the sputum during life and received penicillin treatment, revealed the presence of Streptococcus hemolyticus, and the presence of few non-

pathogenic bacteria. One of the three others which indicated freedom from pathogenic bacteria in life exhibited the presence of a large number of Proteus vulgaris and few colonies of coagulase-positive Staphylococcus aureus. One case yielded type III Pneumococcus pneumoniae and another showed only Streptococcus hemolyticus. Hemophilus influenzae was not isolated from any of these reported cases.

In 1960, Janssen (21) inoculated 10 day old embryonated eggs with a mixture of PR8 influenza virus and Staphylococcus aureus via the allantoic sac and incubated them at 37° C. for 10 days. Embryos infected with the viral and bacterial mixture underwent significantly higher mortality rate than those inoculated with either of the two agents alone. This synergism was maintained even with a very small bacterial-viral inoculum. A high mortality rate was observed in the infected embryos when the bacteria were introduced as late as 24 hours after the establishment of the viral infection. Nevertheless, no synergism was noticed when the viral infection was superimposed 24 hours after the establishment of the bacterial infection. When a combination of serum-neutralized PR8 influenza virus and Staphylococcus aureus was injected into the allantoic sacs of the embryos, no response was detected. Inoculation of the embryonated eggs with a mixture of virus and heat or streptomycin-treated bacteria considerably decreased the synergistic action.

This reduction was small in comparison with that which follows the use of inactivated virus in the mixed inoculum. In 1961, Seller, Schulman, Bouvier, McCune, and Kilbourne (27) observed that mice infected with non-mouse adapted Asian strain of influenza A virus suffered an impaired capacity to remove or destroy staphylococci introduced by respiratory route.

In 1963, Buddingh (6) reported on an investigation of certain aspects of the population dynamics of H. influenzae type b in 14 day embryonated eggs with and without influenza type C virus. He observed that the virulence of the bacterial strains employed as measured by LD_{50} for embryonated eggs was reflected in the proportion of high, medium, and low precipitinogen producers as determined in 30 clone samples. Populations composed of large proportions of high or medium precipitinogen producers were found to be more virulent than those composed of high proportions of low precipitinogen producers. The superimposition of H. influenzae on the viral infection was followed by an acceleration of the bacterial growth rate during the first 6-10 hours as compared with that in embryos infected with the bacteria alone. Furthermore, in the combined infection the bacterial population composition remained constant during the first 6-10 hours. Under these circumstances, during the early hours of infection, the bacterial populations were characterized

by a predominance of high and medium precipitinogen producing individuals. Precipitinogen production and capsule formation by these micro-organisms go hand in hand. Encapsulated bacilli are virulent in the sense that they have the capaticy for invasion of tissues and the blood stream.

In 1963, Jassen, Chappel, and Gerons (22) exposed guinea pigs for 60 minutes to aerosols of influenza virus and/or Staphylococcus aureus. A higher mortality rate and earlier deaths were encountered in the group treated with a combined mixture than those exposed to either of the two agents alone. Reduction in the exposure time or decrease in the concentration of the bacterial or viral elements in the inoculum resulted in a higher survival rate. Such a change in the number of deaths was more sensitive to the fluctuation in the viral dose rather than that of the bacteria. Inactivation of the bacteria by vibration, ultraviolet radiation or heat did not alter appreciably the synergistic action, while the inactivation of the virus interferred with this phenomenon. These authors concluded that the total bacterial cells rather than the number of the viable ones is the important factor in bringing about the consequences of dual infection.

MATERIALS AND METHODS

Collection of Amniotic Fluid from Normal and PR8 Influenza Infected Embryonated Eggs

Fourteen-day old embryonated eggs were inoculated via the amniotic sac with PR8 influenza virus. The amniotic fluid was collected 48 hours after inoculation. Samples from each individual harvest were tested for bacterial sterility by culture on blood agar and for the presence of virus by hemagglutination. Sterile amniotic fluids were pooled. The determination of the hemagglutination titer of the pool provided a comparative estimate of the quantity of the virus present. Normal amniotic fluid was processed in the same manner.

Strains of Hemophilus influenzae Type b

Primary cultures of H. influenzae type b were obtained from cases of purulent meningitis from the contagious ward of Charity Hospital of Louisiana at New Orleans. The first subculture from the primary isolation incubated on Levinthal's agar for 18 to 24 hours was suspended in 20 per cent inactivated normal rabbit serum in sterile distilled

water, distributed in ampules and lyopholized in the frozen state. The flame sealed ampules were stored at $-20^{\circ}C$. This provided a uniform comparable source of the bacterial component.

Determination of the Growth Curve of Hemophilus influenzae Type b

Equal amounts of freshly prepared Levinthal's broth and amniotic fluid from PR8 influenza infected embryonated eggs totalling 25 ml. were combined in a 125 ml. Erlenmeyer flask. Normal non-infected amniotic fluid set up in the same manner served as control. One-tenth milliliter of 10^{-7} dilution of over-night Levinthal's broth culture of Hemophilus influenzae type b obtained from the lyopholized state was introduced into each of the two mixtures, and then incubated at $37^{\circ}C$. Measured samples were drawn from each culture and set up in proper dilutions for colony counts in poured agar plates at 2, 4, 6 and 8 hour intervals. In two experiments of the series, the samples were drawn at 3, 6, 9 and 12, and 6, 9, 12, 15, 18, 21 and 24 hours respectively. The number of H. influenzae type b in the initial inoculum was determined in the same manner using 0.1 ml. of its 10^{-6}, 10^{-7} and 10^{-8} dilution in six poured agar plates respectively. The average number of bacteria obtained from the plate counts was converted into number per 1 ml. culture.

Comparative growth curves were then drawn from the data obtained in this manner.

Determination of the Population Composition of Hemophilus influenzae Type b at Early Intervals of the Growth Curve

Preparation of the inoculum.--It was necessary to know the composition of the population in the inoculum of H. influenzae type b to ascertain whether any changes in this respect were brought about by growth in media containing amniotic fluid from embryos infected with influenza virus as compared with growth in media containing normal amniotic fluid. An inoculum with known composition was artificially put together in the following manner: An arbitrarily chosen primary isolate of H. influenzae type b was inoculated into the surface of Levinthal's agar in such a way that well separated, isolated colonies grew out. From this culture 30 separate adjacent colonies (clones) were introduced into separate 10 ml. amounts of Levinthal's broth in 50 ml. Erlenmeyer flasks. After 24 hours incubation at 37°C., the flask cultures were decanted into tubes and centrifuged at 3000 r.p.m. for 40 minutes at 5°C. The supernates were decanted into separate tubes to be tested for their precipitinogen titer. The bacterial sediments were resuspended in sterile skimmed milk, transferred in 1 ml. amounts to screw cap storage vials, identified by number

to match each supernatent and stored in the CO_2 chest at $-40^{\circ}C$. The gel stabilized double precipitation method of Gispen (9) was used to determine the precipitinogen titer of the supernates of the selected clone cultures. This made it possible to identify each as a low, medium and high precipitinogen producing clone. The inoculum for the population composition determination was then composed by combining equal portions of three over-night Levinthal's broth cultures inoculated with a "low," a "medium" and a "high" precipitinogen producing clone respectively that had been suspended in skimmed milk and in storage at $-40^{\circ}C$. This combined culture was then introduced in 0.1 ml. amounts of a 10^{-7} dilution into 25 ml. of media composed of equal proportions of Levinthal's broth and amniotic fluid from virus infected embryos and also into 25 ml. of media composed of equal proportions of Levinthal's broth and amniotic fluid from uninfected embryos. These cultures were then incubated at $37^{\circ}C$. and 0.5 ml. samples were withdrawn at 3, 6 and 9 hour intervals. At these intervals the samples were inoculated in appropriate dilutions onto the surface of several Levinthal's agar plates and incubated over-night at $37^{\circ}C$. This same procedure was also carried out with the combined culture used as inoculum.

Selection of clones for population composition sample.--From plates inoculated at the stated intervals one

was chosen from which 25 to 30 adjacent isolated clones could be separately transferred into 50 ml. Erlenmeyer flasks containing exactly 10 ml. Levinthal's broth and then incubated for 24 hours. Each culture was decanted into a separate tube, centrifuged in the horizontal at 3000 r.p.m. for 40 minutes at 5° C. A comparison of the precipitinogen producing capacity of the colony samples was then made by the use of the spectrophotometer. This method had been found to give results comparable to those obtained with the gel stabilized double diffusion method. It had the advantage of being less laborious and not time consuming.

Comparative Determination of S.S.S. Precipitate by Spectrophotometric Method

One-tenth milliliter of the individual supernate of the over-night culture of Hemophilus influenzae type b was mixed in a test tube with 0.2 ml. of pooled hyperimmune rabbit serum and the tube was placed in a water bath at 37° C. for 5 minutes. The mixture was then diluted to a final volume of 2.8 ml. with normal saline and reincubated in the 37° C. water bath for another 5 minutes. The contents were then shaken vigorously and transferred into an absorbing cell. Comparative readings of the density of the resulting precipitate was made with Coleman Junior Spectrophotometer, at a wave length of 445 mu. Classification of the different clone

samples into high, medium, and low precipitinogen producers was arbitrarily determined from the comparative optical density readings of the resulting turbidities.

Preparation of Hyperimmune Rabbit Serum for Precipitation of S.S.S. of Hemophilus Influenzae Type b

The method described by Fothergill and Chandler (12) was adopted for the production of anti-Hemophilus influenzae type b hyperimmune serum. Rabbits were injected intravenously with live culture of H. influenzae type b at three daily intervals, followed by four days of rest. The initial dose was 0.05 ml. for the first week, followed by weekly increments of 0.25 ml. until the animal could tolerate 2.0 to 2.5 ml. The agglutinin titer of the pooled serum was found to be 1:1280 when tested against suspensions of the bacilli and 1:64 when tested against precipitinogen. Serum pool was distributed in 100 ml. screw cap bottles and stored in the refrigerator (4° C.) until used.

OBSERVATIONS AND RESULTS

The determination of the numbers of Hemophilus influenzae type b per ml. at 2, 4, 6 and 8 hours following inoculation of culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and of Levinthal's broth and amniotic fluid from influenza infected embryonated eggs (V).--Table I presents the results of 10 experiments in which the numbers obtained from colony counts performed at the indicated intervals in the two types of media can be compared. The numbers presented are the logarithms of the means of the numbers of H. influenzae colonies that grew out from the six 0.1 ml. samples plated at each interval. The growth curves in Fig. 1 are constructed from the means of the mean logarithms of the ten experiments.

At the two and four hour intervals no impressive difference between the numbers of this micro-organism in both media can be ascertained. A significant difference in the bacterial growth in (N) and (V) media becomes apparent at the six hour interval and continues to increase until the eight hour period is reached.

TABLE I

THE MEAN LOGARITHM* OF THE NUMBERS OF HEMOPHILUS INFLUENZAE TYPE b PER ML. AT 2, 4, 6 AND 8 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID AND LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS

Exp. No.	Virus HA titer	2 Hours Groups		4 Hours Groups		6 Hours Groups		8 Hours Groups	
		N	V	N	V	N	V	N	V
1	1:256	5.58	5.50	7.12	7.83	10.23	10.57	12.45	12.86
2	"	4.45	4.70	5.87	6.57	8.08	8.95	11.29	11.83
3	"	4.54	4.42	6.24	6.54	8.62	8.73	10.99	11.26
4	"	4.08	4.23	6.37	6.68	9.74	10.30	12.11	12.64
5	1:128	3.86	3.19	6.37	5.80	8.78	8.50	10.64	11.06
6	"	3.60	3.60	5.87	6.40	8.41	9.16	11.06	11.78
7	"	3.82	3.74	6.33	6.41	8.79	9.03	11.33	11.69
8	"	3.27	3.20	6.13	6.17	6.81	8.61	10.90	11.67
9	"	4.05	4.01	6.68	7.04	9.09	9.61	11.13	12.82
10	1:64	3.04	2.74	7.04	6.73	9.80	10.12	13.18	13.40
Mean		4.03	3.93	6.40	6.61	8.86	9.36	11.51	12.10

*Natural logarithm.

N = Levinthal's broth and normal amniotic fluid.

V = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs.

HA = Hemagglutination.

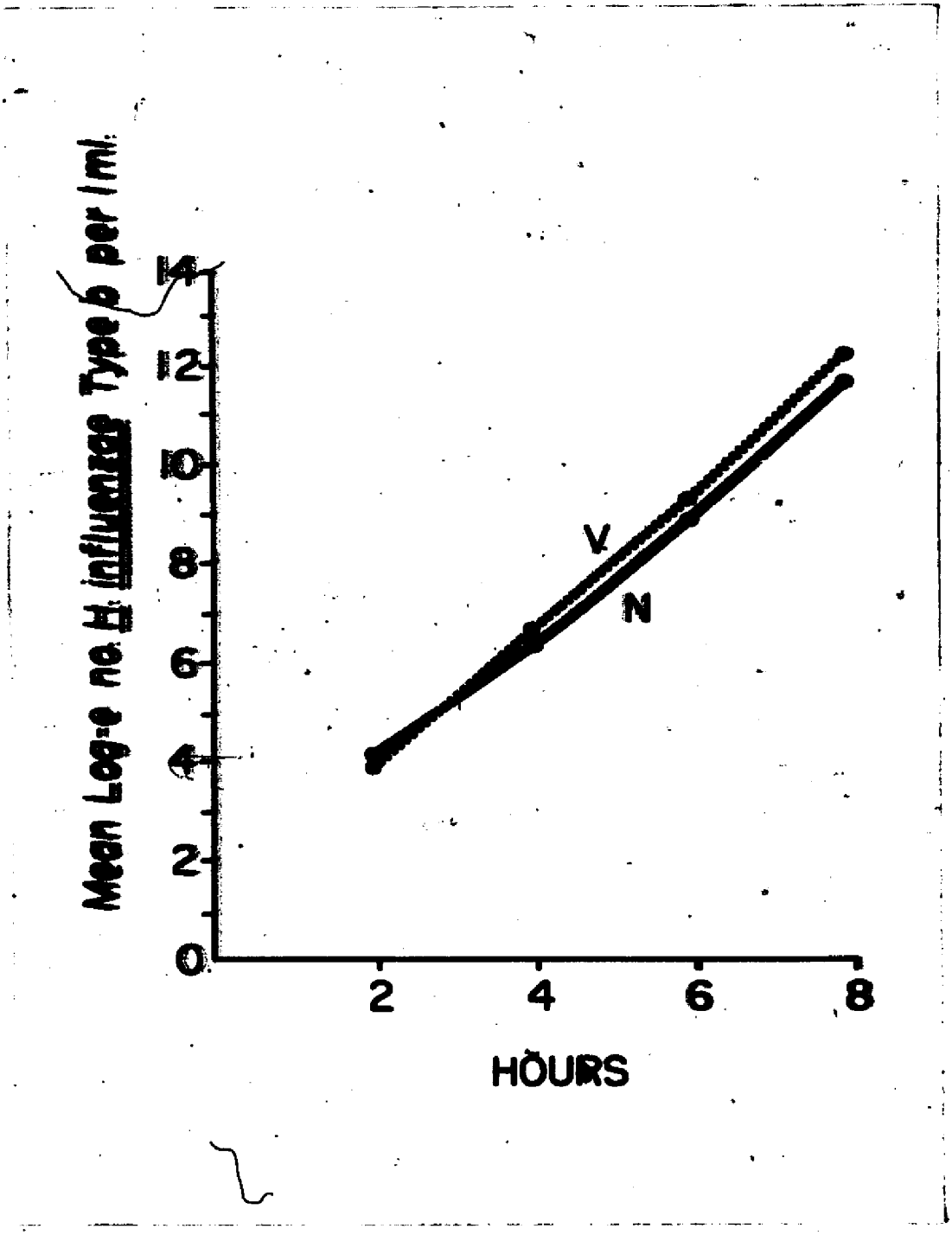

Figure 1. Growth curves of Hemophilus influenzae type b at 2, 4, 6 and 8 hours in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and Levinthal's broth and amniotic fluid from influenza infected embryonated eggs (V).

Table II presents statistical support for the validity of the assumption that the growth rate of H. influenzae is accelerated in media (V) as compared to the growth in media (N). At the two hour period two observations out of ten (experiments 2 and 4) indicated that the numbers of H. influenzae in media (V) are greater than that in media (N). One observation out of ten exhibited equal numbers in both media. In seven out of ten observations there were larger bacterial numbers in media (N) than in media (V). The difference between the bacterial numbers in both media during the first two hours of incubation very likely reflects the variation in the numbers of micro-organisms in the inocula. However, at the four hour interval, there is an increase in the frequency of the greater numbers of H. influenzae in (V) as compared to (N) media. Eighty per cent of the total observations show greater bacterial numbers in (V) than (N) media. At the six hour period, ninety per cent and at the eight hour interval 100 per cent of the total observations showed greater bacterial growth in media (V).

The differences in slopes of the curves of H. influenzae in (N) and (V) media are not restricted to the early hours of growth. Table III and Figures 2 and 3 are based on observations made in two separate experiments in which the numbers of H. influenzae per ml. in both media were determined at 3, 6, 9 and 12 hour periods and 6, 9, 12, 15, 18, 21 and 24 hour

TABLE II

COMPARISON OF THE GROWTH RATE OF *HEMOPHILUS INFLUENZAE* TYPE b AT 2, 4, 6 AND 8 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID (N) AND LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS (V)

Exp. no.	2 Hours	4 Hours	6 Hours	8 Hours
1	−	+	+	+
2	+	+	+	+
3	−	+	+	+
4	+	+	+	+
5	−	−	−	+
6	0	+	+	+
7	−	+	+	+
8	−	+	+	+
9	−	+	+	+
10	−	−	+	+
Per cent (+)	20	80	90	100

\+ = (V > N).

0 = (V = N).

− = (V < N).

TABLE III

LOGARITHM* OF THE MEAN NUMBER OF HEMOPHILUS INFLUENZAE TYPE b PER ML. AT 3, 6, 9, 12, 15, 18, 21 AND 24 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID (N) AND LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS (V)

Exp. no.	HA titer	Groups	3 Hours	6 Hours	9 Hours	12 Hours	15 Hours	18 Hours	21 Hours	24 Hours
11	1:32	N	4.79	8.86	12.82	16.79				
		V	4.91	9.51	13.80	17.53				
12	1:64	N		9.09	12.21	16.12	19.11	21.45	22.06	22.24
		V		10.31	15.05	19.83	21.96	22.87	22.85	22.87

*Natural logarithm.

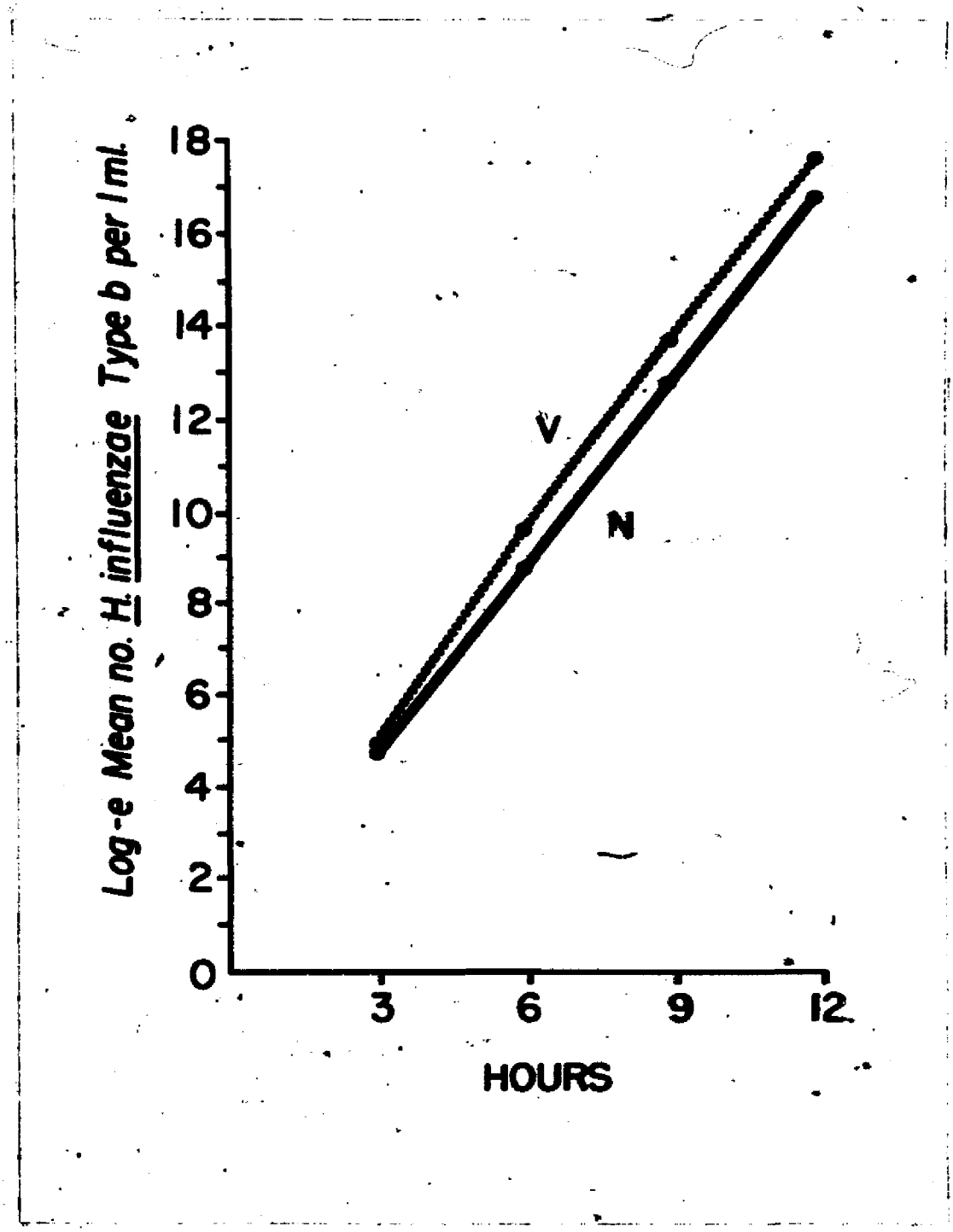

Figure 2. Growth curves of Hemophilus influenzae type b at 3, 6, 9 and 12 hours in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and Levinthal's broth and amniotic fluid from influenza infected embryonated eggs (V).

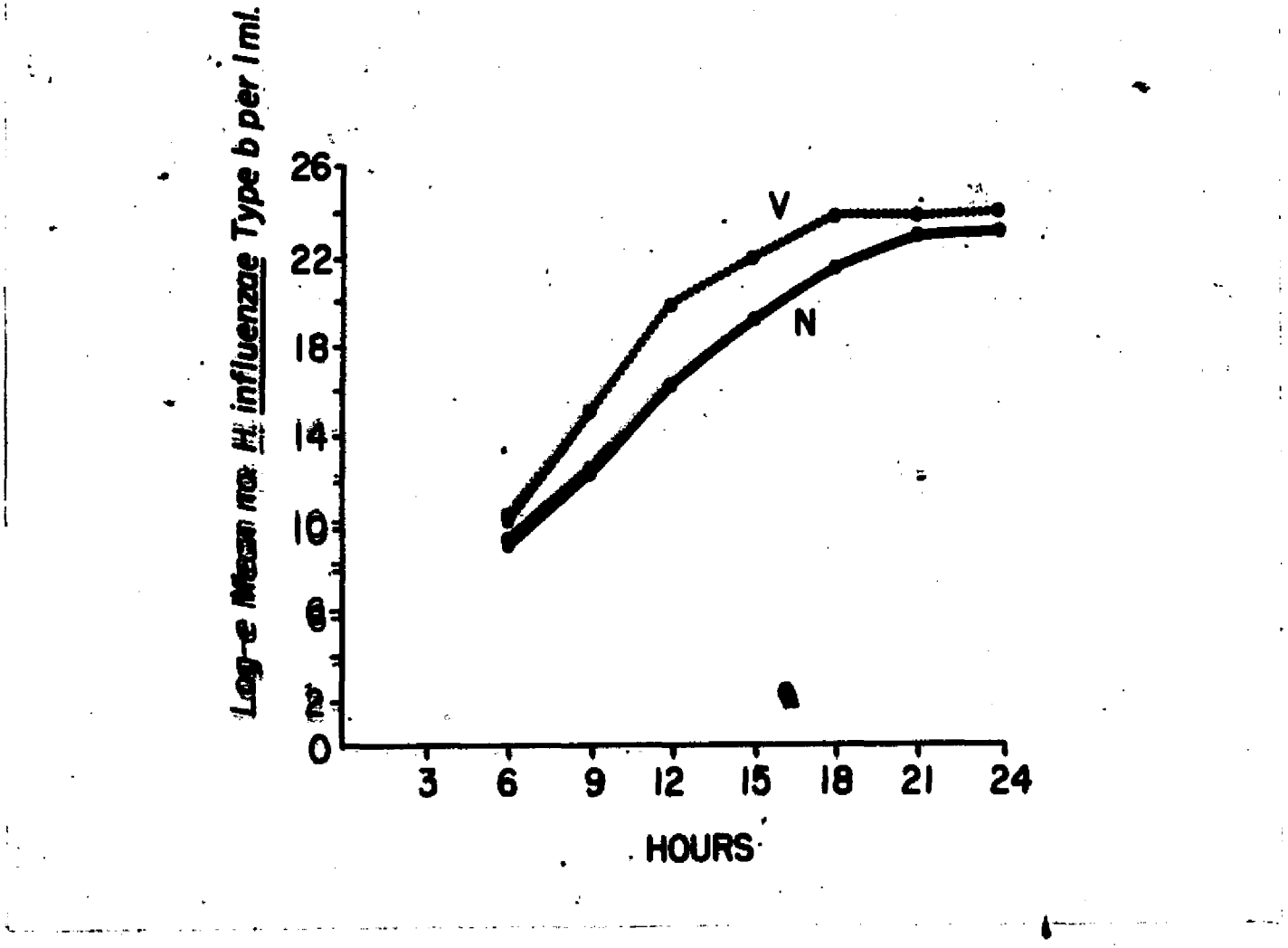

Figure 3. Growth curves of Hemophilus influenzae type b at 6, 9, 12, 15, 18, 21 and 24 hours in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and Levinthal's broth and amniotic fluid from influenza infected embryonated eggs (V).

intervals. The more rapid increase in bacterial numbers in media (V) is clearly apparent. The difference between the two declines gradually after the twelve hour period. The leveling out of both curves around the eighteen hour period indicates the approach of the stationary phase as the number of viable micro-organisms approach one anbther. At the twenty-one hour period, the stationary phase is reached and the numbers in both media become still closer. At the twenty-four hour interval, the growth rates in each of the media appear to be stabilized. The fact that the two curves approach each other at the stationary phase without intersection is an important observation that will be discussed later.

The determination of the presence or absence of growth inhibitory factors for Hemophilus influenzae type b in normal amniotic fluid.--Approximately equal numbers of H. influenzae were inoculated into 25 ml. of media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and into the same amount of straight Levinthal's broth (L). Appropriately diluted samples were set up for colony counts at 2, 4, 6 and 8 hour intervals. The ln values of the counts per ml. of media are presented in Table IV and Figure 4. A definite increase in the growth rate of H. influenzae in media (N) over media (L) is readily observed. Although some of the irregularities may be attributed to sampling errors, the

TABLE IV

THE LOGARITHM* OF THE MEAN NUMBER OF *HEMOPHILUS INFLUENZAE* TYPE b PER ML. AT 2, 4, 6, AND 8 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID AND LEVINTHAL'S BROTH ALONE

Exp. no.	2 Hours		4 Hours		6 Hours		8 Hours	
	Groups		Groups		Groups		Groups	
	N	L	N	L	N	L	N	L
13	3.90	3.62	7.04	5.77	9.81	9.31	13.18	11.95
14	.....	.....	.....	.....	8.39	6.59	11.23	9.65
15	.....	.....	.....	.....	6.75	3.91	9.73	6.91

*Natural logarithm.

N = Levinthal's broth and normal amniotic fluid.

L = Levinthal's broth alone.

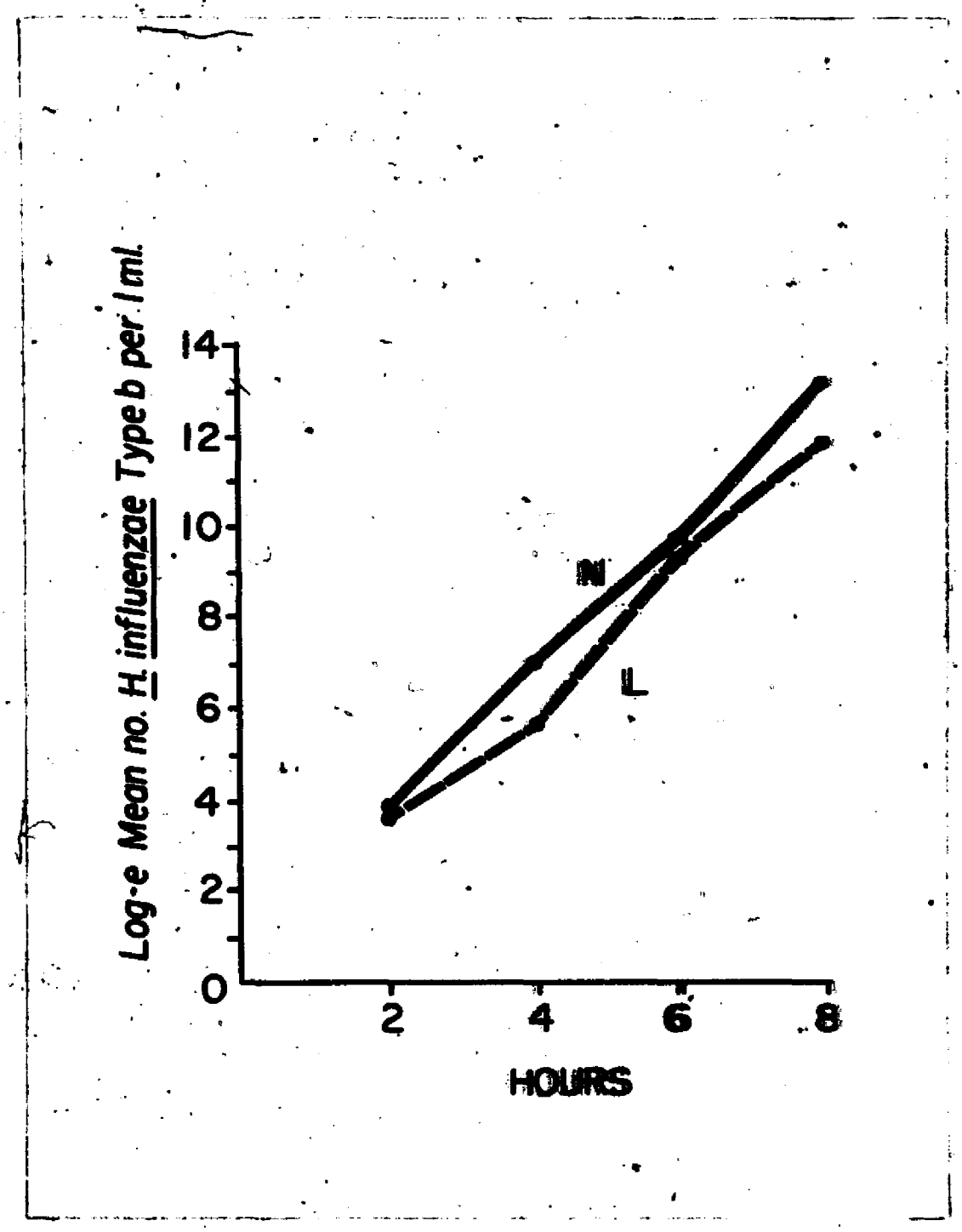

Figure 4. Growth curves of Hemophilus influenzae type b at 2, 3, 6 and 8 hours in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and Levinthal's broth alone (L).

general trend of the curves indicates that H. influenzae multiplies more rapidly in media (N) than in media (L). It may be concluded therefore, that normal amniotic fluid does not contain growth inhibitory factors for this micro-organism.

The determination of the presence or absence of a growth enhancing factor for Hemophilus influenzae type b supplied by influenza virus per se.--Approximately equal numbers of H. influenzae were inoculated into 25 ml. of media consisting of equal parts of Levinthal's broth and amniotic fluid from influenza virus infected embryonated eggs (SV) and into the same amount of media consisting of Levinthal's broth and equal parts of the same amniotic fluid from which the virus had been removed by hemadsorption (SO). Colony counts were made with appropriately diluted samples withdrawn from each culture at 2, 4, 6 and 8 hour intervals. Table V presents the ln values of the number of bacteria per ml. culture at each of these intervals. Figure 5 presents the growth curves obtained in media (SV) and (SO) drawn to the means of the mean ln values of the bacterial number per ml. of culture at each time interval obtained in six experiments.

No significant differences in growth rate of H. influenzae in the two media could be ascertained. The biostatistical evaluation of these data (see Appendix) supports this

TABLE V

THE MEAN LOGARITHM* OF THE NUMBERS OF HEMOPHILUS INFLUENZAE TYPE b PER ML. AT 2, 4, 6 AND 8 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS FROM WHICH THE VIRUS HAS BEEN REMOVED BY HEMADSORPTION AND LEVINTHAL'S BROTH AND SIMILAR AMNIOTIC FLUID TO WHICH THE VIRUS HAS BEEN ADDED

Exp.	HA titer		2 Hours		4 Hours		6 Hours		8 Hours	
			Groups		Groups		Groups		Groups	
no.	Original	Final	SO	SV	SO	SV	SO	SV	SO	SV
16	1:256	1:128	4.08	4.23	6.37	6.68	9.74	10.30	12.11	12.64
17	1:128	1:64	2.83	2.64	7.28	7.57	9.61	9.70	12.21	11.60
18	1:128	1:64	3.45	3.74	6.41	6.78	9.03	9.12	11.39	12.29
19	1:128	1:64	3.27	3.20	6.49	6.17	8.79	8.61	12.42	11.70
20	1:128	1:128	3.88	4.01	7.12	7.04	9.68	9.67	12.76	12.82
21	1:64	1:32	3.81	2.74	6.76	6.73	9.90	10.12	13.13	12.82
	Mean		3.55	3.43	6.74	6.83	9.46	9.59	12.34	12.31

*Natural logarithm.

Viral HA titer for SO is zero in all experiments.

SO = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption.

SV = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs, treated like SO, and to which the virus has then been added.

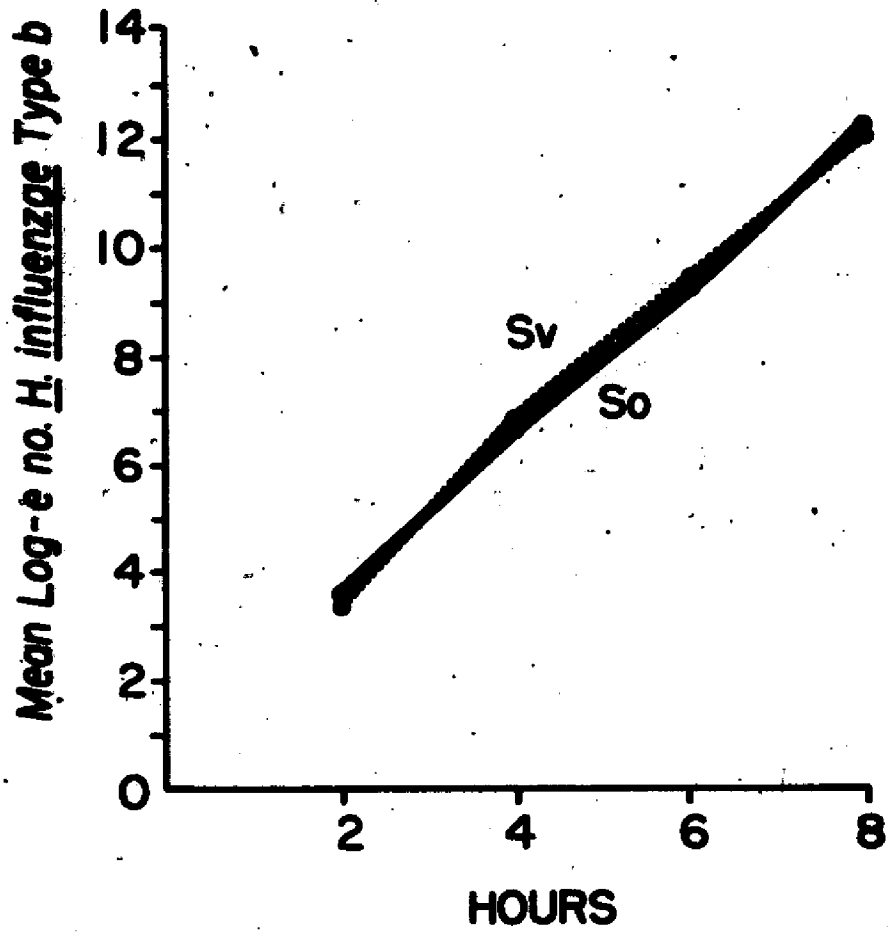

Figure 5. Growth curves of Hemophilus influenzae type b at 2, 3, 6 and 8 hours in culture media composed of equal parts of Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption (SO) and Levinthal's broth and similar amniotic fluid to which the virus has been added (SV).

conclusion. It is thus unlikely that the growth enhancing factors in the combined infection are present in or closely bound to the influenza virus particles.

The validity of this conclusion was further tested by means of the following experiment: Virus was removed by hemadsorption from measured samples of amniotic fluid obtained from influenza virus infected embryonated eggs and added to the same amount of normal amniotic fluid that was then mixed with equal parts of Levinthal's broth (NV). The virus free amniotic fluid obtained in this manner was mixed with equal amount of Levinthal's broth (SO). Both media were inoculated with approximately equal numbers of H. influenzae and incubated at 37° C. Appropriately diluted samples were set up for colony counts at 6 and 8 hour intervals. The number of colonies grown from these samples is presented in Table VI as the ln values for the mean numbers of H. influenzae per ml. culture. These values and the curves presented in Figure 6 indicate that the growth enhancing factors are present in the amniotic fluid from virus infected eggs and does not reside in the virus particles per se. The significant difference between the two slopes is supported by statistical analysis (see Appendix).

TABLE VI

THE MEAN LOGARITHM* OF THE NUMBERS OF HEMOPHILUS INFLUENZAE TYPE b PER ML. AT 6 AND 8 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHSL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS FROM WHICH THE VIRUS HAS BEEN REMOVED BY HEMADSORPTION AND LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID TO WHICH THE VIRUS HAS BEEN ADDED

Exp. no.	HA titer		6 Hours		8 Hours	
	Original	Final	Groups		Groups	
			SO	NV	SO	NV
22	1:128	1:128	8.01	7.79	10.36	10.29
23	1:128	1:64	9.01	7.72	11.84	10.37
24	1:128	1:128	9.94	8.45	13.16	10.94
25	1:128	1:128	9.53	6.22	13.43	9.62
26	1:64	1:32	9.92	9.30	13.48	12.51
27	1:64	1:32	11.77	11.14	15.20	14.11
	Mean		9.70	8.44	12.91	11.31

*Natural logarithm.

Viral HA titer for SO is zero in all experiments.

SO = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption.

NV = Levinthal's broth and normal amniotic fluid to which the virus has been added.

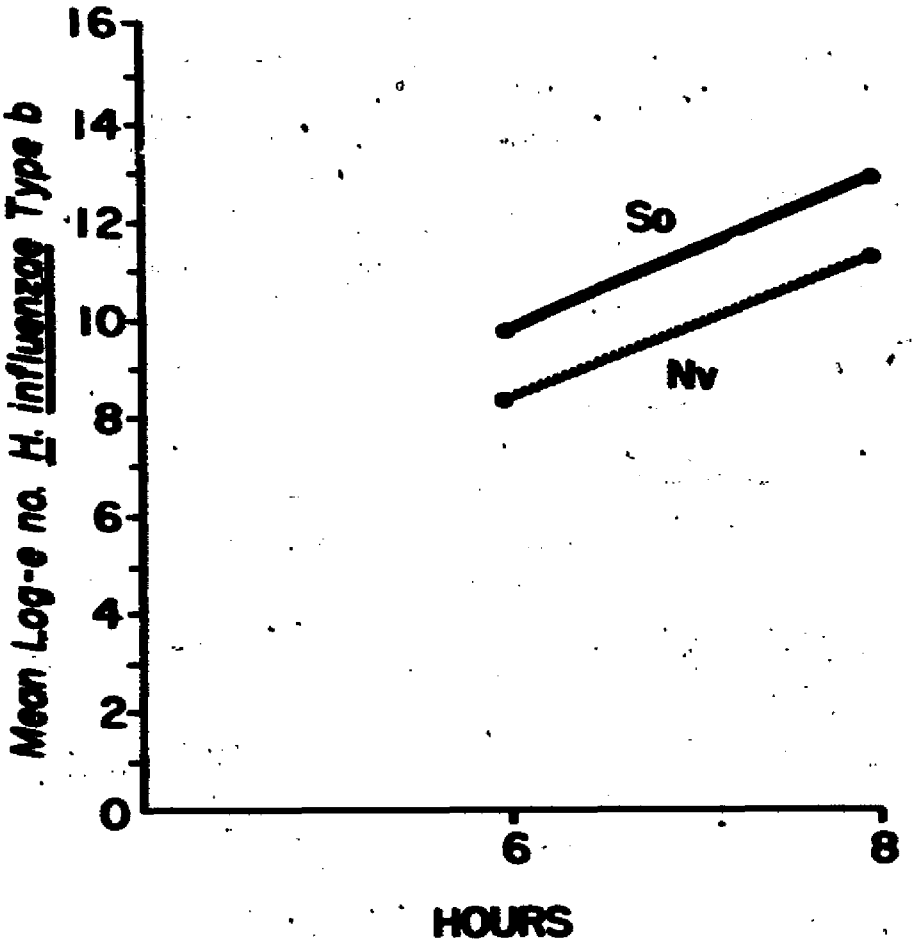

Figure 6. Growth curves of Hemophilus influenzae type b at 6 and 8 hours in culture media composed of equal parts of Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption (SO) and Levinthal's broth and normal amniotic fluid to which the virus has been added (NV).

Comparison of the population composition of Hemophilus influenzae type b cultured in media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and in Levinthal's broth with equal parts of amniotic fluid from influenza virus infected embryonated eggs (V).--It was soon realized that the division of H. influenzae populations into low, medium and high precipitinogen producing clones was an arbitrary one that made no significant distinction. The only sharp distinction that could be made was between clones that produced detectable precipitinogen and those that did not. In the following experiments th distinction between precipitinogen and non-precipitinogen producing clones only will be made.

Table VII and Figure 7 present the results of a representative experiment in which a comparison was made of the population composition of H. influenzae growing in media (N) and media (V) at the 0, 3, 6, 9 and 12 hour intervals following inoculation. The inoculum consisted of 0.1 ml. of a 10^{-7} dilution of a population of H. influenzae type b containing 68.75 per cent precipitinogen producing and 21.25 per cent non-precipitinogen producing clones. In medium (N) the precipitinogen producing clones decreased from 68.75 per cent to 33.33 per cent over the twelve hour period. In medium (V) precipitinogen producing clones increased from 68.75 per cent

TABLE VII

THE PERCENTAGES OF THE PRECIPITINOGEN PRODUCING CLONES IN A POPULATION OF HEMOPHILUS INFLUENZAE TYPE B AT 0, 3, 6, 9 AND 12 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID (N) AND LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS (V)

Exp. No.	HA titer	Groups	0 Hours*			3 Hours			6 Hours			9 Hours			12 Hours		
			No. +	No. =	% +	No. +	No. =	% +	No. +	No. =	% +	No. +	No. =	% +	No. +	No. =	% +
28	1:64	N	22	10	68.75	12	16	42.86	16	17	48.39	9	22	29.03	10	20	33.33
		V	22	10	68.75	22	8	73.33	26	5	83.87	27	6	81.82	28	4	87.50

*Obtained from the estimation of the population composition of the initial bacterial inoculum.

\+ = Precipitinogen producing clone.

\- = Non-producing clone.

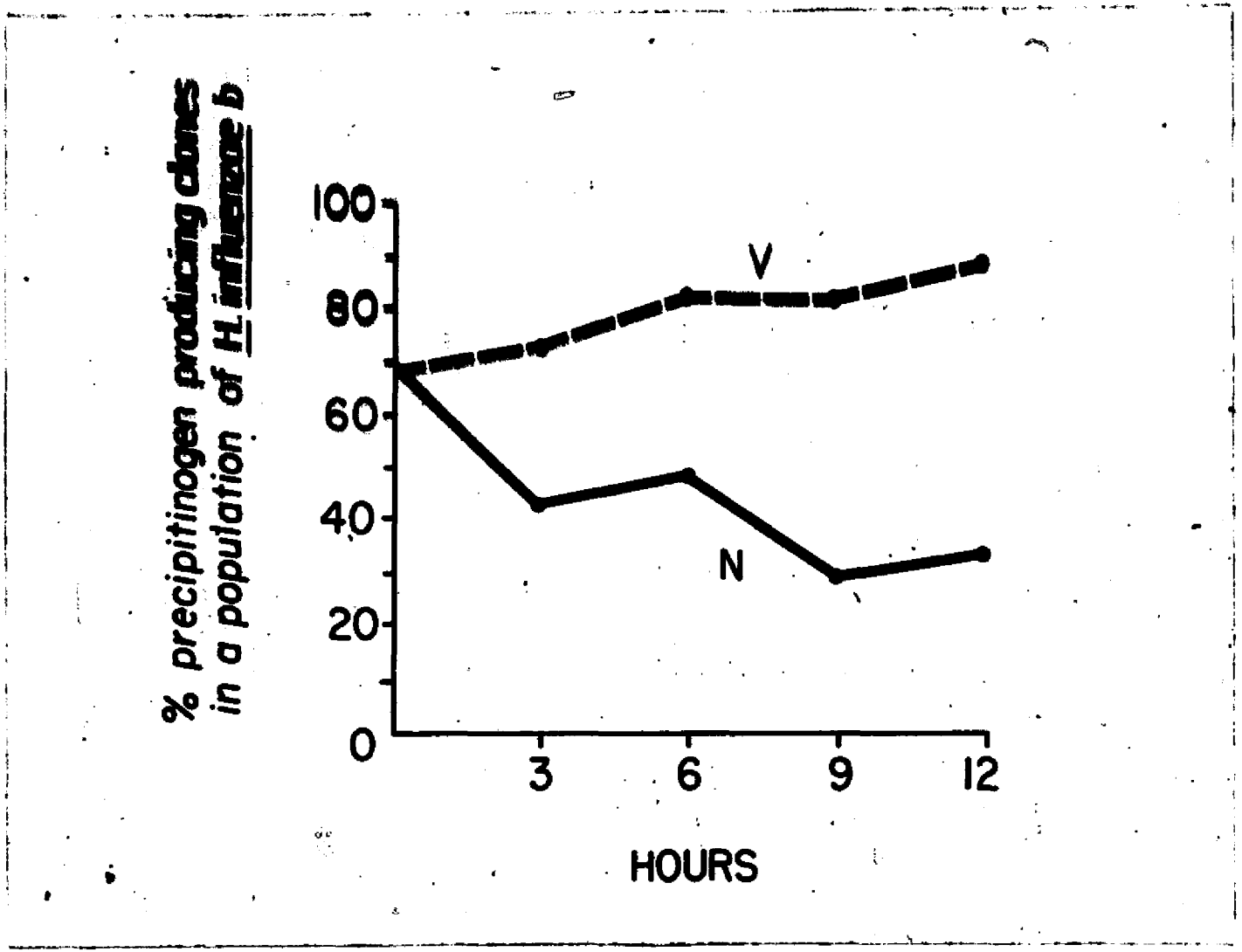

Figure 7. The percentages of precipitinogen producing clones in a population of Hemophilus influenzae type b during 12 hour period in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N) and Levinthal's broth and amniotic fluid from influenza infected embryonated eggs (V).

to 87.50 per cent during the same time intervals. It is apparent that in vitro culture of H. influenzae in media containing amniotic fluid from virus infected eggs promotes the maintenance and increase of the precipitinogen producing, i.e., virulent, bacterial clones as compared to media containing normal amniotic fluid in which the proportion of virulent bacterial elements rapidly decreases.

Determination of the effect of influenza virus per se on the population composition of Hemophilus influenzae type b during the early growth phase.--Virus was removed by hemadsorption from a sample of amniotic fluid obtained from influenza virus infected embryonated eggs. The virus free sample was mixed with equal part of Levinthal's broth (SO). Another culture medium was prepared from equal parts of Levinthal's broth and amniotic fluid identical to that in media (SO) in which the virus had been replaced (SV). The third culture medium was prepared from equal parts of Levinthal's broth and normal amniotic fluid (N). Approximately identical inocula were introduced into each medium. The population composition of the bacteria in each culture was determined at 0, 3, 6 and 9 hour intervals. The results are presented in Table VIII and Figures 8 and 9. Experiment 29 in Table VIII and Figure 8 exhibits a sharp drop in the percentage of the precipitinogen producing clones of H. influenzae grown in medium (V) from 71.45 per cent at 0 hour to

TABLE VIII

THE PERCENTAGES OF THE PRECIPITINOGEN PRODUCING CLONES IN A POPULATION OF HEMOPHILUS INFLUENZAE TYPE b AT 0, 3, 6 AND 9 HOURS IN CULTURE MEDIA COMPOSED OF EQUAL PARTS OF LEVINTHAL'S BROTH AND NORMAL AMNIOTIC FLUID, LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFLUENZA INFECTED EMBRYONATED EGGS FROM WHICH THE VIRUS HAS BEEN REMOVED BY HEMADSORPTION, AND LEVINTHAL'S BROTH AND AMNIOTIC FLUID FROM INFECTED EMBRYONATED EGGS FROM WHICH THE VIRUS HAS BEEN REMOVED BY HEMADSORPTION AND THEN ADDED BY ELUTION

Exp. no.	HA titer Original	HA titer Final	Groups	0 Hours No. +	0 Hours No. −	0 Hours % +	3 Hours No. +	3 Hours No. −	3 Hours % +	6 Hours No. +	6 Hours No. −	6 Hours % +	9 Hours No. +	9 Hours No. −	9 Hours % +
			N	25	8	71.43	11	2	84.62	13	15	46.43	4	26	13.33
29	1:64	0	SO	25	8	71.43	24	3	88.89	25	3	89.29	78	10	88.64
		1:32	SV	25	8	71.43	17	9	65.39	19	5	82.76	25	4	86.21
			N	23	7	76.67	14	13	51.86	13	17	43.33	9	21	30.00
30	1:64	0	SO	23	7	76.67	26	5	83.88	23	7	76.67	35	2	94.60
		1:32	SV	23	7	76.67	22	6	78.58	31	6	86.49	33	0	100.00

Viral HA titer for SO is zero in all experiments.

N = Levinthal's broth and normal amniotic fluid.

SO = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption.

SV = Levinthal's broth and amniotic fluid from influenza infected embryonated eggs treated as SO and to which the virus has been then added.

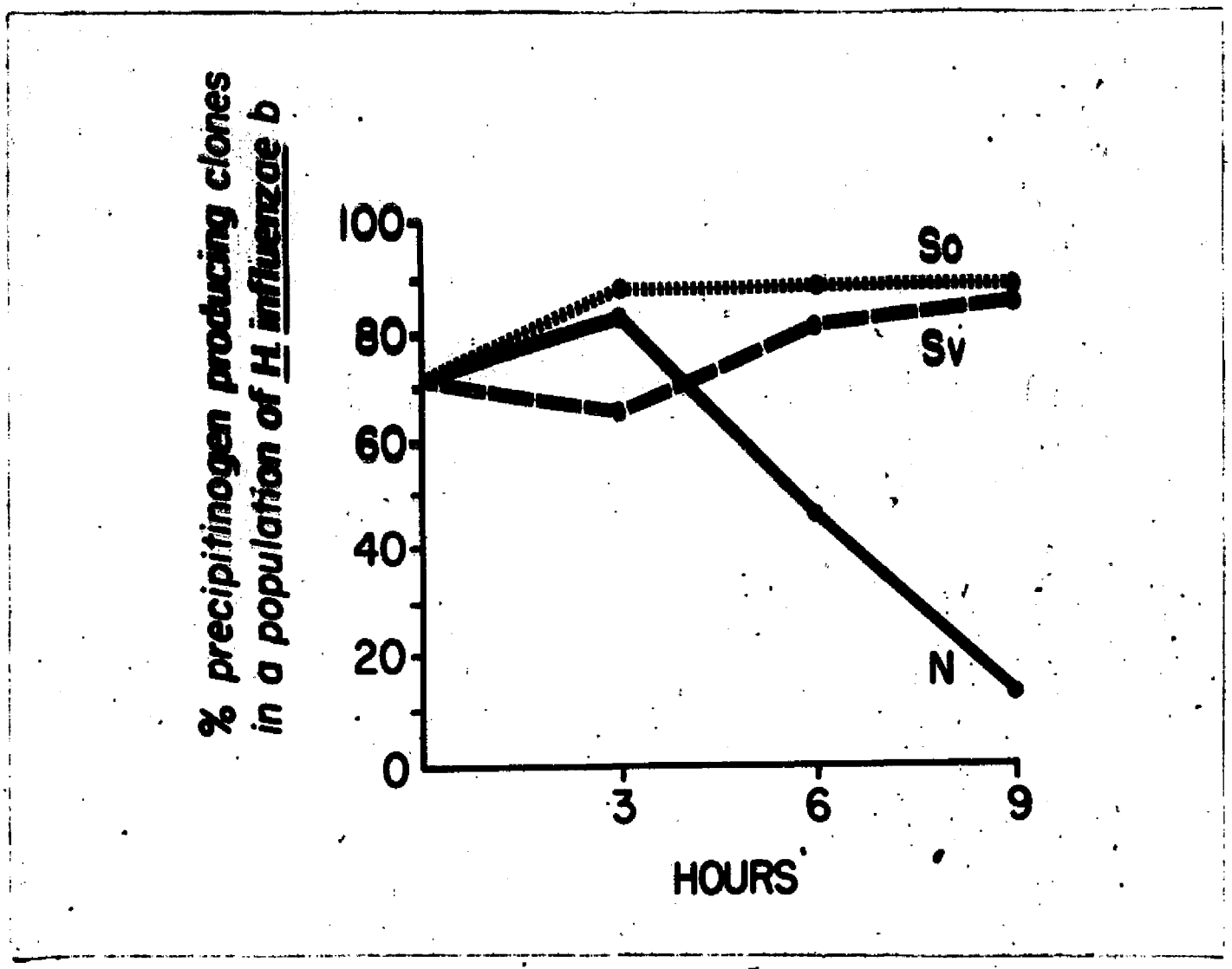

Figure 8. The percentages of the precipitinogen producing clones in a population of Hemophilus influenzae type b during 9 hour period in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N), Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption (SO) and Levinthal's broth and amniotic fluid from infected embryos treated as "S" to which the virus has been then added (SV).

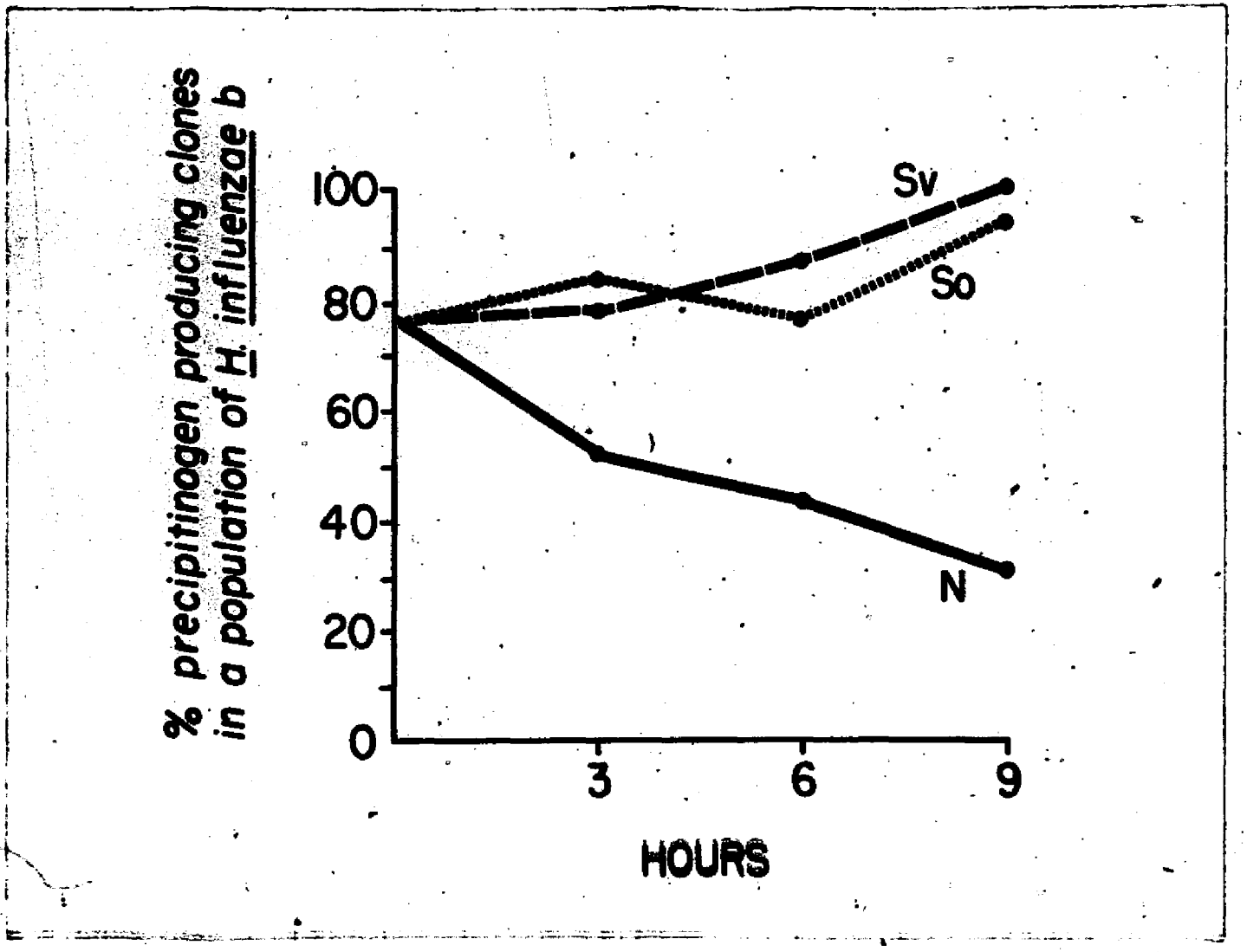

Figure 9. The percentages of the precipitinogen producing clones in a population of Hemophilus influenzae type b during 9 hour period in culture media composed of equal parts of Levinthal's broth and normal amniotic fluid (N), Levinthal's broth and amniotic fluid from influenza infected embryonated eggs from which the virus has been removed by hemadsorption (SO) and Levinthal's broth and amniotic fluid from infected embryos treated as "S" to which the virus has been then added (SV).

13.33 per cent at 9 hour period. In (SO) and (SV)) media the precipitinogen producing clones increased from 71.45 per cent at the 0 hour to 88.89 per cent and 86.21 per cent respectively.

In experiment 30, Table VIII and Figure 9, the precipitinogen producing clones of H. influenzae grown in media (N) decreased from 76.67 per cent at the 0 hour to 30.00 per cent at 9 hour interval, while in (SO) and (SV)) media the proportion of these clones increased from 76.67 per cent at the 0 hour to 94.60 per cent and 100.00 per cent respectively at the 9 hour interval. The results presented in these two experiments indicate that in in vitro, the virus particles by themselves do not influence the population composition of H. influenzae type b and that the factors that maintain and increase the proportion of virulent clones are very likely products derived from the virus infected cells released into the amniotic fluid.

DISCUSSION

The foregoing experiments were conducted to determine if the virulence enhancing effect for Hemophilus influenzae in combined infection with influenza virus could be ascribed to the virus per se or to some factor or group of factors, presumably produced by the virus infected cells of the amniotic membrane of embryonated eggs. It is apparent that the virus as such does not influence the bacterial growth rate, neither does it affect bacterial virulence nor does it seem to have anything to do with maintaining the encapsulated, i.e., virulent, elements of the bacterial population. Whatever this virulence enhancing factor or factors may be, it appears to be present in the amniotic fluid of influenza virus infected embryonated eggs and its presence can be demonstrated by the in vitro methods used in these experiments.

These factors or substances appear to be proteins. Their nature or mode of action in maintaining and enhancing bacterial virulence is still obscure. Preliminary assays by ether, cold alcohol and sodium hydroxide precipitation indicate that there is a much greater quantity of protein in amniotic fluid from virus infected embryonated eggs than in fluid

from normal embryos. This circumstance by itself could account for the fact that the growth curves of H. influenzae in media containing amniotic fluid from virus infected embryos and in media containing normal fluid approach each other during the stationary phase but do not intersect. The availability of more protein during this phase may maintain bacterial multiplication. The additional buffering effect of the protein very likely stabilizes pH and neutralizes staling factors that slow the growth.

The components in amniotic fluid of virus infected embryonated eggs that enhance bacterial virulence in combined infection have not been identified. Until this is accomplished the mechanism of their action is not known and remains the subject of speculation. The stabilization of pH and the neutralization of staling factors may be important at the stationary phase of the growth curve but would not seem to be influential during the early hours of growth when the population dynamics in the direction of increased virulence are most in evidence. This phenomenon most likely involves population competition. Precipitinogen or capsule producing clones appear to possess an inherent, i.e., genetically determined, capacity for extremely rapid multiplication provided the substrates for capsule producing mechanism are available in abundance. It would be important to explore the possibility that complete carbohydrate building blocks are readily avail-

able in amniotic fluid from virus infected embryos. These might be incomplete viral subunits of the ribose conjugated carbohydrate variety in view of the fact that influenza is an RNA virus.

From the genetic standpoint, certain interesting possibilities present themselves. H. influenzae is a highly adapted parasite and is dependent on the host or artificial media for at least the well known V and X factors. Pease and Bisset (24) have suggested for instance that the influenza bacillus may be the evolutionary ancestor of influenza virus by the so called "hypothesis of parasitic degeneration" (Burnet, 7). Some strains of Hemophilus degenerate into mycoplasma or pleuropneumonia-like forms. These L-forms possess the capacity for hemagglutination. Freshly isolated influenza virus strains often exhibit "large bodies" almost identical to those in L-forms of the pleuropneumonia-like group of micro-organisms. Under certain conditions filamentous forms of H. influenzae can be induced that are almost identical with the filamentous forms found in recently isolated strains of influenza virus (24). If this were true, then some of the genetic make-up of the bacterial ancestor may have been handed down to the viral descendent. This genetic determinant may still preserve the code for certain nutritional requirements and thus the bacterium and the virus may share the demand for the same substances. The bacteria

acquire proteins, for instance, through the polymerization of amino acids obtained from the media and the virus through cellular metabolic alteration brought about by the infective virus itself. Thus, the protein or its precursors (amino acids or even polypeptides) may accumulate in the cell and diffuse out or be released by the ruptured cell. Consequently, the bacterium will have access to this nutritional substance that is made ready for it by the infected cell.

This in vitro analysis of the problem of combined viral and bacterial infection supports the hypothesis drawn from the results of the experimental infections of embryonated eggs (5, 6). A selective survival and more rapid growth of the virulent elements of H. influenzae populations can be demonstrated. The conclusion that influenza virus infected cells provide environmental and nutritional conditions that enhance the virulence of H. influenzae populations can be restricted to exclude the virus itself. Further investigation can be directed towards identification and characterization of these factors and the mechanism of their virulence enhancing effect by biochemical analysis of the amniotic fluid of infected embryonated eggs from which the virus particles and hemagglutinating elements are removed by adsorption on erythrocytes and perhaps other biochemical and physical means.

The findings thus far appear to be applicable in explaining the increase in virulence of Hemophilus infleunzae observed in the combined infections studied by Dochez, et al (10) and the synergistic effect on Hemophilus influenzae suis observed by Bang (1, 2). A certain amount of caution must be exercised in applying these explanations to combined infections with other bacterial species such as the streptococci, pneumococci and staphylococci (3, 4, 8, 9, 10, 13, 14, 16, 17, 18, 19, 21, 22, 23, 26, 27, and 29). These micro-organisms differ greatly from Hemophilus influenzae in mechanisms of pathogenicity. Although the role of factors produced by virus infected cells cannot be ignored, the complications introduced by streptococcal and staphylococcal exotoxins and variations in virulence between pneumococcal types emphasize the necessity for analysing these host-parasitic relationships as problems in their own right.

The possible elucidation of some of the mechanism or cycle of events that operate in combined viral and bacterial infection would be of considerable importance in obtaining further insight into certain basic biological phenomenon. Infectious disease is usually considered as the product of the balance between two contenders, the host and parasite. That three contenders may in certain instances be involved and that the establishment of disease in the host can result from the interaction between two parasite populations presents

new problems regarding host-parasite relationships. Furthermore, the possibility of identifying particular factors related to the viral parasite and/or the reaction between host cells and virus would provide a better understanding of the pathogenesis of many infectious diseases.

The separation of some of these factors should eventually lead to a better definition of these components that exert influence on the dynamics of the bacterial population that seem to determine their disease producing potentialities. It may even be possible to characterize these components biochemically and thus utilize them in better directed research for the nature of bacterial growth promoting factors. Presumably also the developments of techniques in this direction might uncover mechanisms that would influence viral production either favorably or unfavorably. With the more exact characterization of the factors that operate in enhancing bacterial virulence in the combined infective process, the way may be opened for the discovery of more refined and perhaps more subtle and effective means of therapy.

SUMMARY

Significantly faster growth rates of Hemophilus influenzae type b were observed in inoculated culture media composed of equal parts of Levinthal's broth and amniotic fluid from PR8 influenza virus infected embryonated eggs than in inoculated media composed of equal portions of Levinthal's broth and normal amniotic fluid. Similarly, culture media composed of equal portions of Levinthal's broth and amniotic fluid from infected embryos maintained the proportion of precipitinogen producing (more virulent) clones of H. influenzae, while the proportion of these clones decreased progressively during the early hours of growth in culture media prepared from equal parts of Levinthal's broth and normal amniotic fluid. It was further determined that normal amniotic fluid does not possess growth inhibitory factors for H. influenzae. Moreover, removal of virus by hemadsorption from culture media composed of equal portions of Levinthal's broth and amniotic fluid from influenza virus infected embryonated eggs neither reduced the growth rate nor the proportion of precipitinogen producing clones of H. influenzae. This suggests that growth

enhancing factors are present in the amniotic fluid from infected embryos and not bound to or inherent in the viral particles *per se*. It may thus be concluded that a substance or group of substances liberated from the virus infected cells of the amniotic sac contain the factors that promote the growth and multiplication of the precipitinogen producing or the more virulent clones in the bacterial population.

The methods developed in these experiments provide an opportunity for the more exact characterization of the virulence promoting factors that are produced for bacterial populations by virus infected cells.

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APPENDIX

Results--Normal (N) vs Virus (V) groups

(1) Bacteria counts were made on plates originating from samples selected from each flask at each time period. (Since some samples were contaminated, sample size varied from 3 to 6 samples per flask per time period.)

(2) Counts were converted to natural logarithms (ln) and a mean (ln count) was computed for each flask for each time period (2 flasks x 4 time periods = 8 observations).

(3) The basic experiment was replicated 10 times so that the analysis was based on 80 observations (10 experiments x 2 flasks (treatments) x 4 time periods = 80 observations).

(4) For the Normal (N) group the slope of the "best fitting" straight line was 2.49 ln units per 2 hour time period. The corresponding slope for the Virus (V) group was 2.72 ln units per 2 hour time period.

(5) These slopes appear to be different. The F value associated with variability among slopes was 5.19 ($P < .05$).*

Supernatant (SO) vs Virus Added (SV) groups

(1) Procedures for this series of experiments are identical with those described for comparison of Normal (N) and Virus (V) groups. Samples were selected from each flask at 2, 4, 6 and 8 hours; a mean ln count was computed for each flask (treatment) for each time period giving 8 observations per experiment (2 flasks x 4 time periods = 8 observations). The basic experiment was replicated 6 times giving a total of 48 observations for the analysis (2 flasks x 4 time periods x 6 experiments = 48 observations).

(2) For the Supernatant (SO) group the slope of the best fitting straight line was 2.91 ln units per 2 hour time period. For the Virus Added (SV) group the corresponding slope was 2.94 ln units per 2 hour time period.

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*For a detailed discussion of the method used to make this analysis, see section 12.8 of G. W. Snedecor, Statistical Methods (Fifth edition; Ames, Iowa: Iowa State College Press, 1962).

(3) There is not sufficient evidence to conclude that the slopes of these two lines are really different. The F value associated with variability among slopes is 0.10 (to be significant at the .05 level an F value of 4.12 or larger would have been required).

ANOVA TABLE A

NORMAL vs VIRUS GROUP

Source	df	SS	ms	F
Hours	3	679.05	226.35	838.33
Groups	1	1.90	1.90	7.04
Replications	9	23.07	2.56	9.48
Hours X Groups	3	1.48	.49	1.81
Among slopes	1	1.40	1.40	5.19*
Non-linear	2	.08	.04	.15
Error	63	16.91	.27	
TOTAL	79	722.41		

*Denotes significance at .05 level.

ANOVA TABLE 3

SUPERNATANT (SO) vs VIRUS ADDED (SV)

Source	df	SS	ms	F
Hours	3	513.96	171.32	815.81
Groups	1	.00	.00	0.00
Replications	5	4.03	.81	3.86
Hours X Groups	(3)	(.12)	(.04)	
Among slopes	1	.02	.02	0.10
Non-linear	2	.10	.05	0.24
Error	35	7.34	.21	
TOTAL	47	525.45		

Supernatant (SO) vs Normal and Virus (NV)

(1) Results may be summarized in the following table of mean ln counts:

Treatment Group	6 Hours	8 Hours	Treatment Means
(SO)	9.70	12.91	11.30
(NV)	8.44	11.31	9.87
Hour Means	9.07	12.11	10.59

(2) Treatment means appear to be different; the F value associated with variability among groups is 22.31 ($P < .01$).

ANOVA TABLE C

SUPERNATANT (SO) vs NORMAL PLUS VIRUS (NV)

Source	df	SS	ms	F
Hours	1	55.48	55.48	100.87
Groups	1	12.27	12.27	22.31
Replications	5	41.23	8.25	15.00
Hours X Groups	1	.17	.17	.31
Error	15	8.29	.55	
TOTAL	23	117.44		

AUTOBIOGRAPHY

Abdul Malik Al-Talib was born in 192- in Mosul, Iraq, where he attended grammar school (1933-1941), intermediate school (1941-1944) and preparatory school (1944-1946). In September, 1947, he enrolled in the Higher Teachers College at the University of Baghdad to study biology and received his B.S. with Honor Degree in June, 1951. Upon graduation, he was appointed as a biology teacher in Adadiya Preparatory School in Mosul until September, 1953, when he enrolled at the University of California, Berkeley, as an unclassified graduate student in the Department of Entomology and Parasitology. In September, 1956, he transferred to the Graduate School of Tulane University in New Orleans to study parasitology and obtained his M.S. degree in June, 1958. Between that time and September of 1960, when he was readmitted to Tulane University, he was employed by several medical firms as medical technologist. In September, 1961, he entered the Graduate School at Louisiana State University to work toward his Ph.D. degree in the Department of Microbiology.

EXAMINATION AND THESIS REPORT

Candidate: Abdul Malik Al-Talib

Major Field: Microbiology

Title of Thesis: An In Vitro Analysis of The Population Dynamics of *Hemophilus Influensae* In Combined Infection With Influenza Virus

Approved:

S. John Buddingh
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April 30, 1964